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RESEARCH PAPER

# A microsatellite study on outcrossing rates and contamination in an *Eucalyptus globulus* breeding arboretum

RAO Hong-xin[1], Briony Patterson[2], Brad Potts[2], Réne Vaillancourt[2]

[1]*Hunan Provincial Forest Botanic Garden, Changsha 410116, P. R. China;*
[2]*School of Plant Science and Cooperative Research Centre for Forestry, University of Tasmania, Private Bag 55, HOBART 7001, Tasmania, Australia*

**Abstract**: Four pairs of microsatellite molecular polymorphism primers were used to analyse microsatellite fingerprints of 188 seedlings derived from an open-pollinated progeny grafted *Eucalyptus globulus* breeding arboretum in Victoria, south-eastern Australia. The microsatellite loci chosen for this study were highly polymorphic with the mean number of alleles per locus of 14.25. Individual mothers varied in their outcrosssing rate estimate from 15% to 95%, the overall outcrossing level in the arboretum was 47.9% and the contamination rate was 17.6%. The high selfing level was likely to result in marked inbreeding depression in the performance of open-pollinated seed lots. Open-pollinated seeds collected from such arboreta are not advisable because of its low genetic quality, although such arboreta may be useful for the seed production through large-scale manual pollination or collecting seeds only from trees or genotypes within the arboretum that have high outcrossing rates.

**Keywords**: *E. globulus*; breeding arboretum; microsatellite; outcrossing rate; pollen contamination

## Introduction

Eucalypts are extensively grown in industrial plantations in the world. The global eucalypt plantation area was estimated to be beyond 17.8 million ha in 2000 (FAO 2000). In China, the *Eucalyptus* plantation estate is estimated to increase by 10% every year (Yang et al. 2006). *Eucalyptus globulus* is one of the main eucalypts grown in pulpwood plantations in temperate regions of the world (Potts et al. 2004). In China, approximate 48 000 ha of *E. globulus* (Potts et al. 2004) are grown mainly in Yunnan Province for fuel wood and oil production (Zang et al. 1995; Doughty 2000). Most plantations are derived from seed-derived plants, while some clonal deployment of the species can be found in countries such as Spain and Uruguay (Potts et al. 2007).

Improved *E. globulus* seeds are typically derived from open-pollinated seedling (Zang et al. 1995) or grafted (Patterson et al. 2004a) seed orchards. One of the main problems existing in open-pollinated *E. globulus* seed orchards is that the species has a mixed mating system and seeds collected from the orchards may contain varying proportions of self-pollinated seeds (Patterson et al. 2004a; Moncur et al. 1995; Russell et al. 2001). Self-pollination may reduce significantly the performance of seedlings grown from such open-pollinated seed lots for being subject to severe inbreeding depression. For example, Hardner et al. (1995) reported that the tree volume growth of self-pollinated plants at age of 43 months was reduced by 48% compared with fully outcrossed progeny. Therefore, it is greatly necessary to establish large-scale manual pollination systems by using grafted arboreta (Harbard et al. 1999; Patterson et al. 2004b). These pollination systems may provide an opportunity for overcoming the inbreeding problem and exploiting specific combining effects. The canopies are maintained accessible through pruning and the use of paclobutrozol, a plant growth regulator retarding growth and promoting flowering (Griffin et al. 1993). In contrasts to open-pollinated grafted seed orchards where ramets of the same genotype are widely dispersed, genotypes are usually grown in line-plots to improve the efficiency of mass pollination. This arrangement is also often used in grafted arboreta established for breeding or archival purposes. As breeding arboreta are often the first facilities establishing newly selected elite germplasm, and usually have transient use for generating crosses for breeding, it is tempting to also use them for open-pollinated seed collection for deployment purposes.

In the present study we used microsatellite DNA markers to study outcrossing rates and pollen flow within a small grafted breeding arboretum of *E. globulus* established by the Southern Tree Breeding Association in Australia using some of the first

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Biography: RAO Hongxin (1970-), corresponding author, female, Associate Prof. in Hunan Provincial Forest Botanic Garden, Hunan, Changsha 410116, P.R. China (E-mail: zwyrhx@163.com)
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selections from the Australian National *E. globulus* Breeding Program. Open-pollinated seeds were harvested from the second flowering season of the arboretum to determine, firstly, whether outcrossing rates were sufficiently high to allow early use of open-pollinated seed from these selected genotypes, and secondly, the extent of pollen contamination occurring at a large mature *E. globulus* plantation about 200 m away from the arboreta.

## Materials and methods

### Material

*Breeding arboretum*

The arboretum studied was established in 1996 and located in Victoria, south-eastern Australia. It comprised grafts of 30 genotypes with between one and four ramets of each genotype surviving at the time of flowering. Ramets of the same genotype were grouped within rows. The spacing between rows was 8 m and within rows was 5 m. Trees had been treated with paclobutrazol to promote flowering. The capsules were harvested from the second flowering season in the arboretum in 2000–2001, when 30 out of 50 trees in the arboretum flowered. Of the 18 genotypes flowered, only nine (represented by 15 trees) had seed capsules in the following year. Genotypes with flowers and capsules were more-or-less randomly spread throughout the arboretum.

*Plant material*

Leaf tissue was collected from all the 30 genotypes presenting in the breeding arboretum and from a further 10 genotypes randomly selected from a nearby stand of *E. globulus* likely as a source of contaminating pollen. Seeds were collected from eight different genotypes. Between 8 and 40 seedlings per genotype were germinated, producing a total of 188 seedlings for DNA fingerprinted.

### Methods

*DNA extraction*

Genomic DNA was extracted from the leaves of candidate parents and whole seedlings following the CTAB method (Doyle et al. 1990) with some modification. Approximately 10 mg of seedling tissue was homogenized with 250 µl of homogenisation buffer (0.1 M Tris pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 0.002 M PVP-40, 0.014 M DDT), then a 250-µl 2 × CTAB buffer was added (4% CTAB, 0.1 M Tris 1.4 M NaCl). Apart from the above modification the protocol was unchanged.

*PCR and microsatellite analysis*

Following extraction, DNA concentration and purity were estimated using agarose gel electrophoresis with ethidium bromide staining. A standard molecular weight marker (Lambda *Hind*III) was used to determine the size and approximate concentration of the DNA. One primer of each microsatellite pair used in this study was synthesized with a fluorescent label (either HEX or TET) attached to the 5' end to allow detection of polymerase chain reaction (PCR) products. "EMBRA10" designed by Brondani et al. (1998), and three "EMCRC" microsatellite primer pairs designed by Steane et al. (2001) were ultimately selected and used throughout the study (refer to Table 1). For each DNA sample, PCR amplification of the four microsatellite loci was performed, with the following PCR reaction mixture (10 µL final volume) containing 10 × BT (1 µL); 10 × BSA (1-2 µL); 2 mM dNTP (0.4-1 µL); 5 µM forward primer (0.4 µL) and 5 µM reverse primer (0.4 µL); 1-2 U-Taq DNA polymerase; 20 ng genomic DNA. 15 mM $MgCL_2$ was used for EMBRA10 and 20 mM for the other three loci. PCR amplification was performed in a Palm cycler (CORBETT RESEARCH) using the touchdown PCR cycling programs shown in Table 1.

**Table 1. Primer sequences and touchdown PCR program condition**

| Locus | Primer sequences | PCR program condition |
|---|---|---|
| EMBRA10 | 5'-GTAAAGACATAGTGAAGACATTCC-3'<br>5'-AGACAGTACGTTCTCTAGCTC-3' | 94°C 3min×1, (94°C 50s, 57°C 50s, 72°C 50s) ×30, (72°C 5min, 10°C 5min) ×1 |
| EMCRC11 | 5'-AACTGACTGTGGATTTGAAGC-3'<br>5'-GTGAGTCATTATTTGGCAACC-3' | 94°C 2min×1, (94°C 50s, 57°C 55s, 72°C 50s) ×10, (94°C 50s, 55°C 55s, 72°C 50s)× 10, (94°C 50s, 53°C 55s, 72°C 50s) ×10, (72°C 5min, 10°C 5min) × 1 |
| EMCRC5 | 5'-GTTTCTTCCTCTGCTTGTTGC-3'<br>5'-GATGGGTTCGGATTTAGGC-3' | 94°C 2min ×1, (94°C 50s, 62°C 55s, 72°C 50s) × 10, (94°C 50s, 60°C 55s, 72°C 50s) × 10, (94°C 50s, 58°C 55s, 72°C 50s) × 10, (72°C 5min, 10°C 5min) × 1 |
| EMCRC6 | 5'-CTTCAAGGTTCACAGATGG-3'<br>5'-TCTTCATAAGTCCCCTAATCA-3' | 94°C 2min ×1, (94°C 50s, 52°C 55s, 72°C 50s) ×10, (94°C 50s, 50°C 55s, 72°C 50s) × 10, (94°C 50s, 48°C 55s, 72°C 50s) ×10, (72°C 5min, 10°C 5min) × 1 |

Following PCR two volumes of formamide loading buffer (formamide with 0.1 mg/mL bromophenol blue sodium salt) were added to each tube and denatured at 94ºC for 3 min before electrophopetic separation on a 15-cm 4% denaturing acrylamide gels at 40°C, using a Gel Scan 2000 Gel Analyzer (Corbett Research, Sydney, Australia). Alleles sizes were recorded using Gs 2000 software by comparison with a 60-400 base standard DNA ladder (purchased from Promega), using the computer software package Gene Profiler (Scanalytics, Inc. Fairfax, VA). Alleles were binned into size increments of two nucleotides, from the smallest allele. Stutter bands were observed, of which the darkest and usually largest band was considered as the true allele. When only one band was observed, homozygosity was assumed to ignore possible null alleles. Microsatellite profiles were successfully obtained from 188 seedlings derived from eight of the nine female genotypes of the capsules harvested.

*Parentage assignment*

Seedlings were classified as the derived from self-pollination if all their alleles matched those of the known mother. If a non-maternal allele was present at any locus the seedling was classified as an outcross. If an outcross seedling contained an allele that was not present in the eighteen genotypes flowered in the arboretum, then it was classified as a contaminant. CERVUS 2.0 (Marshall et al. 1998; Slate et al. 2000) was then used to identify which ones of the 28 candidate parents (all 18 genotypes flowered at the breeding arboretum and the other 10 trees from the neighbouring *E. globulus* stand) were likely pollen parents. CERVUS 2.0 uses a likelihood ratio method that is defined as the likelihood of parentage of a particular individual relative to the likelihood of parentage of an arbitrary individual. Parentage is assigned to a particular individual if the likelihood ratio is large relative to the likelihood ratios of other individuals. When several unlinked marker loci are used for analysis, the likelihood ratios derived at each locus can be multiplied together. The logarithm of the combined likelihood ratio is termed the logarithm of the odds (LOD) score. Marshall et al. (1998) defined the logarithm of the likelihood ratio of the two most-likely males as ⊿ (the difference in LOD scores) and used CERVUS to generate a criterion that allowed assignment of parentage to the most-likely individual with a known level of statistical confidence. In this study, the simulation parameters for CERVUS 2.0 generating ⊿ criteria were set as follows: 10,000 cycles of simulation; 40 candidate parents; 0.01 as the proportion of loci mistyped and confidence levels of 95% and 80%. CERVUS 2.0 was also used to determine the number of alleles (K); observed (Ho) and expected heterozygosity (He); polymorphic information content (PIC); average exclusion probability (Excl); and Null allele frequency estimate (Null freq).

## Results and analysis

Analysis of the allele frequencies

All of the microsatelite loci used had a large number of alleles (mean number of alleles per locus was 14.25), low frequency of null alleles (ranged from 0.03 to 0.05) and high degrees of expected heterozygosity (average He = 0.866) (refer to Table 2). The total exclusionary power was 0.962 for the first parent and 0.994 for the second parent, and was sufficiently high to correctly assign parentage in the majority of cases.

**Table 2. Diversity and exclusion parameters for microsatellite loci**

| Locus | k | Ho | He | PIC | Excl(1) | Excl(2) | Null freq |
|---|---|---|---|---|---|---|---|
| embra10 | 15 | 0.825 | 0.903 | 0.882 | 0.641 | 0.782 | 0.0385 |
| emcrc11 | 11 | 0.795 | 0.850 | 0.830 | 0.530 | 0.696 | 0.0314 |
| emcrc5 | 17 | 0.750 | 0.848 | 0.820 | 0.523 | 0.689 | 0.0501 |
| emcrc6 | 14 | 0.784 | 0.862 | 0.834 | 0.538 | 0.702 | 0.0335 |
| average | 14.25 | 0.789 | 0.866 | 0.839 | 0.962* | 0.994** | |

**Note**: Excl (1) = total exclusionary power (first parent); Excl (2) = total exclusionary power (second parent); K = number of alleles; Ho = observed heterozygosity; He = expected heterozygosity; PIC = polymorphic information content; *= total exclusionary power (first parent); **=total exclusionary power (second parent)

Outcrossing and contamination in the arboretum

Of the 188 seedlings sampled, 90 were assigned as outcrosses, giving an outcrossing rate of 47.9% (Table 3). Individual mothers varied in their outcrosssing rate estimate from 15 to 95%. Of the 90 outcrosses, 33 were classified as being derived from pollination outside of the arboretum. The overall contamination rate was estimated to be 17.6%, but varied from 0 to 95% between families. A total of 67% (12 genotypes) of the 18 genotypes that flowered in the arboretum sired offspring, with the number of outcross pollen donors per genotype ranging from 0 to 6.

This study clearly argued that the average genetic quality of seeds collected from the *E. globulus* arboretum was likely low due to the high rate of selfing and high contamination from the surrounding plantation. The average outcrossing rate of 48% obtained in this arboretum was typically lower than that of native stand with an average outcrossing rate of 90% (Foster et al. 2007), although the outcrossing rate can vary depending on numerous factors such as isolation degree of the tree (Hardner et al. 1996a), seed location collected from the bottom or top of the tree (Patterson et al. 2001; Patterson et al. 2004b), and level of self-incompatibility of the individual trees (Patterson et al. 2004b). According to previous studies, the average open-pollinated *E. globulus* seed orchard estimates were 60% by Patterson et al (2004a.), 77% by Moncour et al. (1995) and 92% by Russell et al. (2001).

**Table 3. Outcrossing and pollen contamination of the open-pollinated offspring in the arboretum**

| Genotypes of mother | Number of seedlings assayed | Outcrossing rate (%) | Contamination rate (%) | Most-likely arboreta parents contributing to outcrossing (number offspring sired) |
|---|---|---|---|---|
| 414 | 20 | 50.0 | 0 | 3981(4), 6263(6) |
| 907 | 40 | 17.5 | 2.5 | 6326(1), 3538(1), 3561(1),3215(1), 3548(1), 6263(1) |
| 3548 | 20 | 20.0 | 0 | 3561(2), 6176(1), 3981(1) |
| 3561 | 8 | 75.0 | 75.0 | |
| 3981 | 40 | 65.0 | 27.5 | 6263(4), 414(6), 3538(1), 6176(2), 6326(2) |
| 6176 | 20 | 75.0 | 25.0 | 3981(8), 6263(2) |
| 6326 | 20 | 95.0 | 40.0 | 907(1), 3548(5), 6891(1), 4157(1), 6176(2), 3561(1) |
| 6891 | 20 | 15.0 | 10.0 | 6176(1) |
| Overall | 188 | 47.9 | 17.6 | |

## Discussion

Numerous factors may, individually or together, explain the poor outcrossing rate in this breeding arboretum. Firstly, only 60% of the trees flowering in the year before may have limited the availability of pollen. Reduced pollen availability may have been compounded with a reduction in pollinator population size and movement. Moreover, low density of flowering trees has been shown to reduce outcrossing rates in native populations of this species (Hardner et al. 1996b) and others (Lee 2000; Obayashi et al. 2002; Fuchs et al. 2003). Secondly, because this arboretum is clonal, what is being detected as self-pollination may in fact include inter-tree pollination between different grafts of the same genotype, in a manner similar to that observed in the clonal plant *Decodon verticillatus* (Fuchs et al. 2003) and a grafted *Eucalyptus globulus* seed orchard (Patterson et al. 2004a). The unique structure of this breeding arboretum, with clones clustered together, may have accentuated selfing. As identical clones will flower at approximately the same time in the flowering season due to the high heritability of this trait (Gore et al. 1995), for a given tree in flower, there will be a relatively high concentration of self pollen in the pollen pool. Thirdly, self-incompatibility levels vary from 0 to 100% in *E. globulus* depending upon genotype (Patterson et al. 2004b; Pound et al. 2002a; Pound et al. 2002b; McGowen et al. 2004a) and the breeding arboretum may be comprised of an atypically high number of self-compatible genotypes. As expected, higher self-compatibility levels have been shown to be associated with higher outcrossing rates in both native stands and seed orchards of *E. globulus* (Patterson et al. 2004b; Patterson et al. 2001). Fourthly, it is also possible that the presence of null alleles has inflated the estimates of selfing. However, estimates of the null alleles frequency in *E. globulus* are low (Hardner et al. 1996a), arguing against this being a significant factor. Fifthly, the exclusionary power of the loci used to detect outcrosses is another factor affecting estimates of outcrossing, and while the presence of non-maternal alleles means that outcrosses are unlikely to be missed, it is possible for maternal allele combinations to arise through outcrossing. However, in the present case, the total exclusionary power for the second parent is high indicating this unlikely becomes a major effect on our estimates.

Pollen contamination from unimproved genetic sources can reduce the expected gains from seed orchards (Eldridge et al. 1993). The pollen contamination rate in this arboretum was lower than other estimates for other forest trees (El-Kassaby et al. 1986; Harju et al. 1996; Lai et al. 1997; Pakkanen et al. 2000; Moriguchi et al. 2002), but still relatively high at 17.6%. In the case of eucalypts, microsatellites studies have estimated the rate of pollen contamination to be 39% for an *E. grandis* orchard (Chaix et al. 2003), 29% for an *E. urophylla* seed orchard (Grattapaglia et al. 2004) and 46% for an *E. grandis* artificial population (Megan et al. 2007).

Strategies should be developed and implemented to reduce pollen contamination. It may be efficient to use buffer zones, such as non-compatible or asynchronously flowering trees, or felling adjacent contaminating sources 2-3 years before seed harvest. Recent studies suggest that, in the case of eucalypts, km's of isolation from compatible pollen sources is required to guarantee low contamination levels, although a significant reduction may be achieved with ordering isolation distances of 300 m (Barbour et al. 2005). However, maintaining high outcrossing rates is still more problematic. Outcrossing rates of individual trees can be relatively stable across seasons (McGowen et al. 2004b), and one possible way of obtaining highly outcrossed seed is to collect only from trees or genotypes within the arboretum that have high outcrossing rates (e.g. genotype 6326), or from trees with high levels of self-incompatibility (McGowen et al. 2004a; Patterson et al. 2004b). But, as a general strategy this option would be expensive as it requires molecular estimation of outcrossing rates or undertakes controlled pollinations to measure self-incompatible levels (Pound et al. 2002a; Pound et al. 2002b). Another option for deploying seed production using such arboreta is to manually pollinate flowers through techniques such as mass supplementary pollination (MSP) (Patterson et al. 2004a). Such manual pollination systems are now widely used with *E. globulus* (Potts et al. 2007).

## References


Barbour RC, Potts BM, Vaillancourt RE. 2005. Pollen dispersal from exotic eucalypt plantations. *Conservation Genetics*, **6**: 253–257.

Brondani RPV, Brondani C, Tarchini R, Grattapaglia D. 1998. Development, characterization and mapping of microsatellite markers in *Eucalyptus grandis* and *E. urophylla*. *Theoretical and Applied Genetics*, **97**: 816–827.

Chaix G, Gerber S, Razafimaharo V, et al. 2003. Gene flow estimation with microsatellites in a Malagasy seed orchard of *Eucalyptus grandis*. *Theoretical and Applied Genetics*, **107**: 705–712.

Doughty RW. 2000. The *Eucalyptus*: A natural and commercial history of the gum tree. Baltimore and London: The Johns Hopkins University Press, p225–228.

Doyle JJ, Doyle JL. 1990. Extraction of plant DNA from fresh tissue. *Focus*, **12**: 13–15.

Eldridge K, Davidson J, Harwood C, Van Wyk G. 1993. Eucalypt Domestication and Breeding. Oxford: Oxford University Press, p332–334.

El-Kassaby YA, Ritland K. 1986. The relation of outcrossing and contamination to reproductive phenology and supplemental mass pollination in a Douglas-fir orchard. *Silvae Genetica*, **35**: 240–244.

FAO. Global forest resources assessment 2000-main report. FAO Forestry paper 140, 2000 [2008-03-07]. Http://www.fao.org/forestry/fo/fra/main/index.jsp.

Foster SA, McKinnon GE, Steane DA, Potts BM, Vaillancourt RE. 2007. Parallel evolution of dwarf ecotypes in the forest tree *Eucalyptus globulus*. *New Phytologist,*, **175**: 370–380.

Fuchs EJ, Lobo JA, Quesada M. 2003. Effects of forest fragmentation and flowering phenology on the reproductive success and mating patterns of the tropical dry forest tree *Pachira quinata*. *Conservation Biology*, **17**:149–157.

Gore PL, Potts BM. 1995. The genetic control of flowering time in *Eucalyptus globulus*, *E. nitens* and their $F_1$ hybrids.Proc. In: BM Potts, NMG Borralho, JB Reid, et al. (eds). CRCTHF-IUFRO Conf. Hobart: CRCTHF-IUFRO, pp.241–242.

Grattapaglia D, Ribeiro VJ, Rezende GD. 2004. Retrospective selection of elite parent trees using paternity testing with microsatellite markers: an alternative short term breeding tactic for *Eucalyptus*. *Theoretical Applied Genetics*, **109**: 192–199.

Griffin AR, Whitemen P, Rudge T, Burgess IP, Moncur Ml. 1993. Effect of paclobutrazol on flower-bud production and vegetative growth in two species of *Eucalyptus*. *Canadian Journal of Forest Research*, **23**: 630–647.

Harbard JL, Griffin AR, Espejo J. 1999. Mass controlled pollination of *Eucalyptus globulus*: a practical reality. *Canadian Journal of Forest Research*, **29**: 1457–1463.

Hardner CM, Potts BM. 1995. Inbreeding depression and changes in variation after selfing *Eucalyptus globulus* subsp. *globulus Silvae Genetica*, **44**: 46–54.

Hardner CM, Vaillancourt RE, Potts BM. 1996a. Stand density influences outcrossing rate and growth of open-pollinated families of *Eucalyptus globules*. *Silvae Genetica*, **45**: 226–228.

Hardner CM, Vaillancourt RE, Potts BM. 1996b. Stand density influences outcrossing rate and growth of open-pollinated families of *Eucalyptus globulus*. *Silvae Genetica*, **44**: 46–54.

Harju AM, Nikkanen T. 1996. Reproductive success of orchard and non-orchard pollens during different stages of pollen shedding in a Scots pine seed orchard. *Canadian Journal of Forest Research,* **26**: 1096–1102.

Lai Huanlin, Wang Zhangrong 1997. Comparison of genetic structure between parents and progeny from a *Masson Pine* seed orchard and a plantation nearby. *Forest Scientific Research,* **10**: 490–494.

Lee SL. 2000. Mating system parameters of *Dryobalanops aromatica* Gaertn. F. (Dipterocarpaceae) in three different forest types and a seed orchard. *Heredity*, **85**: 338–345.

Marshall TC, Slate J, Kruuk LEBI. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**: 639–655.

McGowen MH, Potts BM, Vaillancourt RE, Gore P, Williams DR, Pibeam DJ. 2004a. The genetic control of sexual reproduction in *Eucalyptus globulus*. In: NMG Borralho, JS Periera, C Marques, J Coutinho, M Madeira, M Tome(eds), *Eucalyptus* in a changing world. Portugal: Averio, pp.104–108.

McGowen MH, Williams DR, Potts BM, Vaillancourt RE. 2004b. Stability of outcrossing rates in *Eucalyptus globulus* seedlots. *Silvae Genetica*, **53**: 42–44.

Megan J, Mervyn S, Robert H, Angela D. 2008. Pollen flow in *Eucalyptus grandis* determined by paternity analysis using microsatellite markers. *Tree Genetics & Genomes*, **4**: 37–47.

Moncur MW, Mitchell A, Fripp Y, Kleinschmidt GJ. 1995. The role of honey bees (*Apis mellifera*) in eucalypt plantation forestry. *Commonwealth Forestry Review*, **74**: 350–354.

Moriguchi Y, Taira H, Tsumura Y. 2002. Gene flow of seed orchard in *Cryptomeria japonica* d.don using microsatellite markers. *Plant Animal Genome*, **10**: 562.

Obayashi K, Tsumura Y, Ihara-Ujino T, Niivama K, Tanouchi H, Suyama Y, Washitani I, Lee C, Lee S, Muhammad N. 2002. Genetic diversity and outcrossing rate between undisturbed and selectively logged forests of *Shorea curtisii (Dipterocarpaceae)* using microstatellite DNA analysis. *International Journal of Plant Sciences*, **163**: 151–158.

Pakkanen A, Nikkanen T, Pulkkinen P. 2000. Annual variation in pollen contamination and outcrossing in a *Picea abies* seed orchard. *Scandanavian Journal of Forest Research*, **15**: 399–404.

Patterson B, Peter G, Potts B, et al. 2004a. Advances in pollination techniques for large-scale seed production in *Eucalyptus globulus*. *Australian Journal of Botany*, **52**: 781–787.

Patterson B, Vaillancourt RE, Pilbeam D, Potts BM. 2004b. Factors affecting variation in outcrossing rate in *Eucalyptus globulus*. *Australian Journal of Botany*, **52**: 773–780.

Patterson B, Vaillancourt R, Potts B. 2001. Eucalypt seed collectors: beware of sampling seedlots from low in the canopy. *Australian Forestry*, **64**: 139–142.

Potts BM, Vaillancourt RE, Jordan G, Dutkouski G, Silva J, Mckinnon G, Steane D, Volker P, Lopez G, Apiolaza L, Li Y, Marques C, Borralho N. 2004. Exploration of the *Eucalyptus globulus* gene pool. In: N Borralho, JS Pereira, C Marques, J Cotinho, M Madeira, M Tome (eds). Eucalyptus in a changing world. Portugal: Aveiro, pp.46–61.

Potts B, McGowen M, Williams D. 2007. Advances in reproductive biology and seed production systems of *Eucalyptus*: The case of *Eucalyptus globulus*. In: Book of abstracts of IUFRO conference—Eucalypts and Diversity: Balancing Productivity and Sustainabilty, Durban, South Africa, pp.22–26.

Pound LM, Wallwork MAB, Potts BM, Sedgley M. 2002a. Early ovule development following self- and cross-pollinations in *Eucalyptus globulus* Labill. ssp. *globulus*. *Annals of Botany*, **89**: 613–620.

Pound LM, Wallwork MAB, Potts BM, Sedgley M. 2002b. Self-incompatibility in *Eucalyptus globulus* ssp. *globulus* (Myrtaceae). *Australian Journal of Botany*, **50**: 365–372.

Russell J, Marshall D, Griffin R, Harbard J, Powell W. 2001.Gene flow in South American *Eucalyptus grandis* and *Eucalyptus globulus* seed orchards. In: TUFRO symposium—developing the eucalypt of the future. Chile, Valdivia: pp.149–150.

Slate J, Marshall TC, Pemberton JM. 2000. A retrospective assessment of the accuracy of the paternity inference program Cervus. *Molecular Ecology*, **9**: 801–808.

Steane DA, Vaillancourt RE, Russell J, Powell W, Marshall D, Potts BM. 2001. Development and characterisation of microsatellite loci in *Eucalyptus globulus* (Myrtaceae). *Silvae Genetica*, **50**: 89–91.

Yang Minsheng, Wu Zhihua, Chen Shaoxiong. 2006. Ecological Effect of Eucalyptus Forest and Its Ecological Forest Management. *Eucalypt Science & Technology*, **23**: 32–39.

Zang D, Wang H, You Y. 1995. Performance and selection of a 4-year *Eucalyptus globulus* seedling seed orchard in Yunnan, China. In: B.M. Potts, N.M.G. Borralho, J.B. Reid et al. (eds), Eucalypt Plantations: Improving Fibre Yield and Quality. Hobart, Tasmania: Proc. CRCTHF-IUFRO, pp.226–229.